IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	: Rene Gantier et al.	Art Unit	: 1631
Serial No.	: 10/658,355	Examiner	: Russell Scott Negin
Filed	: September 8, 2003	Conf. No.	: 3519
Cust. No.	: 20985		
Title	: RATIONAL DIRECTED PROTEIN EVOLUTION USING TWO-DIMENSIONAL RATIONAL MUTAGENESIS SCANNING		

DECLARATION PURSUANT TO 37 C.F.R. §1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Manuel Vega, declare as follows:

1) I am a joint inventor of the above-captioned application. I am founder of Nautilus Biotech, which is the Applicant in the above-described application. I am currently Chief Executive Officer of Nautilus Biotech. I have held this position since January 2000. Prior to that time, I held senior management positions at Microgen SA from 1992 to 1996 and at Gènèthon from 1997-1999. I also was an adjunct Professor of Human Gene Therapy at the Universidad Nacional del Sur (UNS) from 1992-1997. I also have gained scientific research experience in Germany at Würzburg University, in France at the Institut National de la Santé et de la Recherche Médicale (INSERM), in the Netherlands at the Netherlands Organization for Applied Scientific Research (TNO) and in Argentina at Consejo Nacional de Investigaciones Cientificas y Técnicas (CONICET).

2) I received a Licentiate degree in Biological Sciences from the Universidad Nacional del Sur (UNS), Bahia Blanc, Argentina in 1982. I received a Doctor in Biology from UNS in 1987. I have over 20 years of experience in the field. I have authored or co-authored over 26 publications in scientific journals and book chapters, and I have received numerous awards for my scientific achievements.

3) I am familiar with the outstanding Office Action and methods described in the cited references.

4) The method described in the above-captioned application is directed to a semi-rational directed evolution of proteins based on the *in silico* identification of amino acid positions

or regions associated with a predetermined activity or property, followed by modification with replacement amino acids. This method, referred to as a 2-dimensional method (2-D), efficiently and reliably identifies modified polypeptides, particularly therapeutic polypeptides, that are altered in a predetermined property. The method described in the above-captioned application permits the design and identification of polypeptides containing only slight variations (including those having only one or two amino acid changes) that exhibit a predetermined property, such as a property of the polypeptide, and retain a desired activity, such as a therapeutic activity. Because the method reliably results in such identification and permits identification of mutants with a predetermined property based on changes in a small number of amino acid residues, it is a powerful method, compared to any in the cited references.

5) As described in the above-captioned application, the method is more powerful and more efficient than other directed evolution approaches. The method described in the above-captioned application is based on scanning amino-acid replacement using a "half-predictive and half-random" approach. Based on the property to be evolved, the target positions (is-Hits) to be modified are identified by *in silico* methods and include all target positions associated with the particular predetermined property or activity being evolved. The replacing amino acid types are selected by *in silico* methods to be those predicted to be amenable to producing a predetermined property or activity. The modified proteins are produced and screened one-by-one so that the identity of each is known *a priori*. The method, thus, provides an unbiased approach that is efficient and powerful in its capacity for discovering modified polypeptides having a change in a predetermined property with a minimal number of amino acid modifications. Also, the ability to generate each mutant polypeptide one-by-one for screening in addressable arrays permits their ease of identification and contributes to the efficiency of the method.

6) Using the methods claimed in the above-captioned application, Nautilus Biotech has identified many modified polypeptides evolved to exhibit a predetermined property or activity. The method has been practiced on proteins that occur in diverse protein families. The predetermined properties or activities of candidate polypeptides include, for example, conformational stability, thermal tolerance and protease resistance. Included among such candidate polypeptides are those containing one or two amino acid mutations, such that the predetermined property is altered, but a native activity is retained.

7) Among such candidate polypeptides identified by the methods claimed in the above-captioned application are those that are resistant to proteases. In many instances, by virtue of only a single amino acid change, the polypeptides exhibit increased protease resistance. As described in co-pending U.S. application No. 10/658,834, filed the same day as the above-captioned application, therapeutic polypeptides, which normally must be administered via injection, when modified to exhibit increased protease resistance, can be administered orally. LEAD polypeptides having increased resistance to protease have been identified for a variety of therapeutic proteins, including Interferon-alpha, Interferon-beta, Interferon-gamma, Growth Hormone, Factor IX, erythropoietin and High Mobility Group Box One (HMGB1) Box A. Such polypeptides exhibit increased resistance of the entire molecule to proteolysis in blood, tissue and the intestine. By virtue of the resistance to proteases, the proteins can be administered at lower doses for subcutaneous administration compared to other established therapeutic proteins. In addition, as described in the co-pending U.S. Application No. 10/658,834, proteins exhibiting increased resistance to proteases can be successfully administered orally. Hence, among the advantages provided by the methods in the above-captioned application, is the reliable discovery of polypeptides, particularly therapeutic polypeptides, containing only a few modifications in the primary sequence, particularly one or two modifications, that can be formulated for oral administration without specific formulation requirements except for the protein itself.

8) Using the methods as claimed in the above-referenced application and standard methods as described herein, myself and other scientists involved in these projects have prepared more than a thousand candidate LEAD polypeptides. In particular, LEAD polypeptides exhibiting the predetermined property of increased protease resistance have been identified. Included among these LEAD polypeptides are variants of Interferon-alpha and Growth Hormone that are in clinical development for subcutaneous administration and oral administration.

As evidence of the potential of the methods described in the above-captioned application, a description of the methods and results obtained for exemplary polypeptides identified based on the claimed methods for the predetermined property of protease resistance follows. The data are not meant to cover the full scope of polypeptides identified using the methods claimed in the above-captioned application, but are provided to evidence the power of the method to reliably identify candidate polypeptides based upon a predetermined property or activity.

I. Methods

A. Resistance to Proteolysis

The following protocol to assess resistance to proteases was used for all proteins tested, unless otherwise indicated.

Mutants were treated with proteases in order to identify resistant molecules. The relative resistance of the mutant proteins compared to the native protein against enzymatic cleavage was determined by exposure to a mixture of proteases (containing 1.5 pg of each of the following proteases (1% wt/wt, Sigma): α-chymotrypsin, carboxypeptidase, endoproteinase Arg-C, endoproteinase Asp-N, endoproteinase Glu-C, endoproteinase Lys-C, and trypsin) at 25°C for a set time period between 30 minutes to 120 minutes. At the end of the incubation time, 10 µl of anti-proteases complete medium containing mini EDTA free tablets, Roche (one tablet was dissolved in 10 ml of DMEM and then diluted to 1/1000) was added to each reaction in order to inhibit protease activity. Treated samples were then used to determine residual activity such as anti-viral or proliferative activity.

For erythropoietin, the relative resistance of the mutant proteins compared to the native protein against enzymatic cleavage was determined by exposure to a 1.5 % protease mixture (wt/wt) containing each of the following proteases α-chymotrypsin, Endoproteinase GluC and trypsin (Sigma) or by exposure to a 3 % protease mixture (wt/wt) containing each of the following proteases, α-chymotrypsin, Endoproteinase GluC and trypsin (Sigma). The protease reaction was processed as described above and residual activity was determined.

B. Anti-viral activity

Residual activity of Interferon-alpha and Interferon-gamma variant polypeptides were assessed in an anti-viral assay. Anti-viral activity can be measured by cytopathic effects (CPE). Anti-viral activity of variant polypeptides was determined by the capacity of the cytokine to protect HeLa cells against EMC (mouse encephalomyocarditis) virus-induced cytopathic effects. The day before, HeLa cells ($2x10^5$ cells/ml) were seeded in flat-bottomed 96-well plates containing 100 µl/well of Dulbecco's MEM-Glutamax-sodium pyruvate medium supplemented with 5% SVF and 0.2% of gentamicin. Cells were grown at 37°C in an atmosphere of 5% CO_2 for 24 hours.

Two-fold serial dilutions of variant polypeptide samples preincubated with protease were made with MEM complete media into 96-deep well plates. Twenty-four (24) hours after seeding the cells, the medium was aspirated from each well and 100 µl of diluted samples were added to

HeLa cells. Each sample dilution was assessed in triplicate. The last two rows of the plates were filled with 100 µl of medium without added sample in order to serve as controls for cells with and without virus. After 24 hours of growth, a 1/1000 EMC virus dilution solution was placed in each well, except for the cell control row. Plates were returned to the CO_2 incubator for 40 - 48 hours. The medium was discarded, and the cells were washed twice with 100 µl of 1X PBS and stained for 1 hour with 80- 100 µl of staining solution (typan blue or ehanol-formamide-methyl blue mixture) to determine the proportion of intact cells. Plates were washed in a distilled water bath and the cell-bound dye was extracted using 80 - 100 µl of ethylene-glycol mono-ethyl-ether (Sigma). The absorbance of the dye was measured using an ELISA plate reader (Spectramax; Molecular devices) at 660 nm.

C. Cell Proliferation Activity

1. Nb2-11 cells

Residual activity of Growth Hormone variants were tested by assessing their effects on the proliferation of the rat lymphoblast Nb2-11 cells. Nb2-11 cells were cultured in Fisher medium supplemented with 10% of SVF and 10% of equine serum (ES). 24 hours before the proliferation assay, the cells were centrifuged and washed with PBS. The cells were then cultured in Fisher medium supplemented only with 10% of ES at a density of 0.5 - 0.8 x 10^6 cells/ml. After 24 hours of culture the cells were seeded in 96 well plates at 4 x 10^4 cells/well and treated with three fold serial dilution of pre-treated native human growth hormone (hGH) or mutants between 6000 pg/ml and 0.3 pg/ml in triplicates. NIBSC hGH was used as an internal control for each proliferation assay.

After 48 hours of treatment with either native or mutant hGH (pre-treated with proteases) the proliferation of Nb2-11 cells was measured. 20 µl of Cell Titer 96 AQ (Promega) per well was added and cells incubated for 1 hour at 37°C. The assay measures the conversion of the tetrazolium MTS into a soluble formazan. Samples were read in a Spectramax reader (Molecular Device) at 490 nm.

2. TF-1 cells

Residual activity of erythropoietin (EPO) variants were tested by assessing their effects on the proliferation of human erythroleukemia cells (TF-1 cell line). TF-1 cell line was maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% FCS, 2mM L-glutamine and 2ng/ml of human recombinant GM-CSF at 37°C in a humid atmosphere with a composition

of 7% CO_2 /95% air in T175 (175cm2) polystyrene tissue culture flask and split two times per week. Twenty four hours before use in proliferation assays, cells were washed two times in ice cold PBS and re-suspended for 16 hours in GM-CSF free RPMI medium supplemented with 2mM glutamine and 10% FCS.

TF-1 cells were plated into 96-well plates at $4x10^4$ cells per well in 70µl of GM-CSF free RPMI medium supplemented with 2mM glutamine and 10 % FCS. Each aliquot samples were subjected to a two-fold serial dilution into 96-Deep-well plates and EPO dilutions (30µl) were added to each well containing 70µl of TF-1 cells with a final concentration ranging from 70000 to 34.2 pg/ml. Each EPO sample dilution was assessed in triplicate. No GM-CSF was added to the last row ("G" row) of the flat-bottomed 96-well plates in order to evaluate basal absorbance of non proliferative cells. A 2-fold serial dilution (70000 to 34.2 pg/ml) of internal positive controls including both the second international standard for EPO (NIBSC, 88/574)) and the first international standard for GM-CSF (NIBSC, 88/646) were also performed and added in triplicate to plate assay in order to standardize proliferation results.

The plates were incubated for 48 hours at 37°C in a humidified, 7% CO_2 atmosphere. After 48 hours of growth, 20 µl of Cell titer 96 Aqueous one solution reagent (Promega) was added to each well and incubated 3 hours at 37°C in an atmosphere of 7% CO_2. To measure the amount of colored soluble formazan produced by cellular reduction of the MTS, the absorbance of the dye was measured using an ELISA plate reader (spectramax) at 490nm.

D. Subcutaneous administration in Cynomolgus Monkeys

Naïve cynomolgus monkeys (*Macaca fascicularis*; one male and one female for each subgroup) received subcutaneous (SC) injections of 0.3 mg/kg native IFN-α, Intron A® (a commercially available IFN-α), or selected LEAD IFN-α mutant polypeptides. At various time points (pre-dose, 0.02 0.5, 1, 2, 4, 8, 12, 16, 24, 48, 72 h post-dose), blood was collected into anti-coagulant and anti-protease solution and the residual anti-viral activity was determined as described above.

E. Per-os (PO; oral route) administration to Cynomolgus Monkeys

A single dose (0.9 mg/kg) of enteric-coated capsule formulation of mutant interferon-alpha (IFN-α) and native IFN-α were tested after PO administration in Cynomolgus monkeys (*Macaca fascicularis*) in order to compare the pharmacokinetic and systemic profile of the two

polypeptides after oral administration. A total of four purposely bred Cynomolgus monkeys, two males and two females, divided into two groups with one male and one female per group, were dosed with 0.9 mg/kg of an enteric-coated capsule formulation of mutant IFN-α or native IFN-α by PO route. At various time points (day -1, 0, 0.5, 1, 1.5, 2, 4, 6, 8, 12, and 24 h) post-administration, a blood sample (1 ml) was taken from the saphenous or cephalic veins of all monkeys for the determination of the remaining anti-viral activity levels of mutant IFN-α or native IFN-α in plasma. Each animal was checked for mortality and clinical signs at least twice a day during the treatment period. The body weight of each animal was recorded at least twice during the pre-treatment period.

II. Results

The methods as claimed in the above-captioned application have been applied to thousands of polypeptides based upon the predetermined property of protease resistance. Exemplary results are provided below for some of the polypeptides prepared and identified using the methods as claimed.

A. In Vitro Resistance to Proteolysis

Residual activity (either anti-viral or proliferative activity) was determined for exemplary candidate LEAD polypeptides identified by the claimed method and native polypeptides following treatment with protease mixture. In the Tables, the resistance to proteolysis is indicated as "no change" or "increased" as compared to the residual activity of the respective native polypeptide under the same protease treatment conditions. The data are not meant to be representative of all proteases, but are exemplary data showing the resistance to proteolysis to an exemplary protease cocktail as described in the methods above. Thus, the data are not comprehensive and are not meant to be indicative that other polypeptides do not exhibit protease resistance.

1. Interferon-alpha

a. Primary Selection

Using the methods as claimed in the above-captioned application, 184 variants of interferon-alpha (IFN-α2b) were generated and tested based on the predetermined property of increased protease resistance. Candidate LEADs were tested *in vitro* for protease resistance by incubating 100 µl of 1500 pg/ml (500 U/ml) of IFNα-2b with a cocktail of proteases as described

above. Following protease treatment, residual activity was assessed by an anti-viral assay. Table 1 shows the results of some of the tested polypeptides. The results show that many of the polypeptides tested exhibited an increased resistance to protease *in vitro* as compared to native IFN-α.

TABLE 1: Interferon Alpha LEADS	
Mutant	**Resistance to proteolysis**
F27V	No change
R33H	No change
E41Q	Increased
E41H	No change
E58Q	Increased
E58H	Increased
E78Q	Increased
E78H	Increased
Y89H	No change
E107Q	Increased
E107H	Increased
P109A	No change
L110V	No change
M111V	No change
E113H	Increased
L117V	Increased
L117I	Increased
K121Q	Increased
R125H	Increased
R125Q	Increased
K133Q	Increased
E159H	Increased
E159Q	Increased

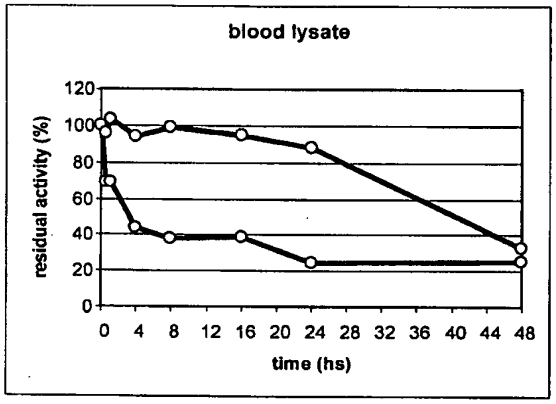
b. Kinetic Analysis

One variant polypeptide (termed mutant IFN-α herein) was chosen for further kinetic analysis. The decreased susceptibility to proteases was evaluated by exposing mutant IFN-α and native IFN-α to the following proteolytic treatments: a) human blood lysate; b) human serum; c) chymotrypsin (10% w/w); d) a protease mixture (1% w/w of α-chymotrypsin, carboxypeptidase, endoproteinase Arg-C, endoproteinase Asp-N, endoproteinase Glu-C, endoproteinase Lys-C and trypsin) as described above in the methods for assaying resistance to proteases, except that incubation with protease mixture was done for variable time. After the incubation, the protease reaction was stopped and the residual anti-viral activity was determined as described above. The results are shown below in Figure 1, A) – D). The results show that mutant IFN-α (blue line)

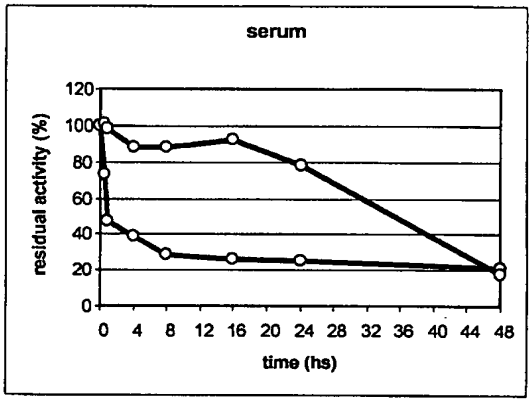
maintained 50 – 90% of its antiviral activity for up to 24 hours, compared to native IFN-α (black line), which lost greater than 30% activity within 1 hour and maintained only 25-30% of its activity at 24 hours.

FIGURE 1:

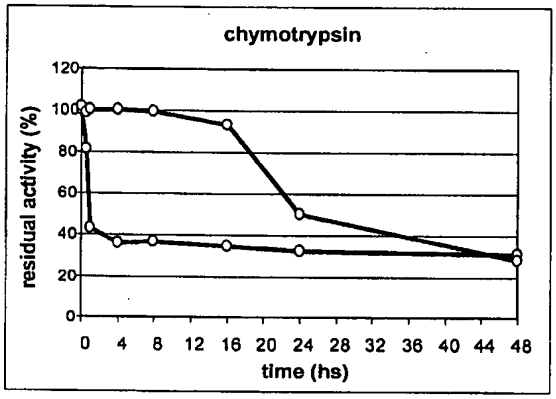
A.



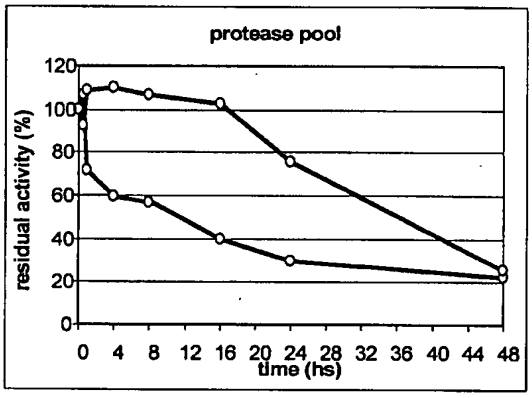
B.



C.



D.



2. Growth Hormone

Using the methods as claimed in the above-captioned application, 222 variants of human Growth Hormone (hGH) were generated and tested based on the predetermined property of

increased protease resistance. Candidate LEADs and SuperLEADs of hGH were tested *in vitro* for protease resistance by incubating 15 ng of native hGH or variants with a cocktail of proteases as described above, followed by assessment of residual proliferative activity on Nb2-11 cells as described above. Table 2 shows the results of some of the tested polypeptides. The results show that many of the polypeptides tested exhibited an increased resistance to protease *in vitro* as compared to native hGH.

TABLE 2: Growth Hormone LEADS and SuperLEADs

Mutation	Resistance to proteolysis	Mutation	Resistance to proteolysis
F1I/P2A	increase	F92V	no changes
F1I/P2S	no changes	L93I	no changes
F1V/P2A	no changes	L93V	no changes
F1V/P2S	no changes	R94H	no changes
P5A	no changes	R94Q	no changes
P5S	increase	F97I	no changes
L6I	no changes	F97V	no changes
L6V	no changes	L101I	no changes
R8H	no changes	L101V	increase
R8Q	no changes	Y103H	no changes
L9I	no changes	Y103I	no changes
L9V	increase	D107N	no changes
F10I	no changes	D107Q	no changes
F10V	no changes	Y111H	no changes
D11N	increase	Y111I	increase
D11Q	no changes	D112N	increase
M14I	no changes	D112Q	no changes
M14V	increase	L113I	no changes
L15I	no changes	L113V	no changes
L15V	no changes	L114I	no changes
R16H	increase	L114V	no changes
R16Q	no changes	K115N	no changes
R19H	no changes	K115Q	no changes
R19Q	no changes	D116N	no changes
L20I	no changes	D116Q	increase
L20V	no changes	L117I	no changes
L23I	increase	L117V	no changes
L23V	increase	E118Q	no changes
F25I	no changes	E118H	no changes
F25V	no changes	E118N	no changes
D26N	increase	E119Q	increase
D26Q	no changes	E119H	no changes
Y28H	no changes	E119N	no changes
Y28I	no changes	L124I	no changes
E30Q	no changes	L124V	increase

TABLE 2: Growth Hormone LEADS and SuperLEADs

Mutation	Resistance to proteolysis	Mutation	Resistance to proteolysis
E30H	no changes	M125I	increase
E30N	no changes	M125V	increase
F31I	no changes	R127H	no changes
F31V	no changes	R127Q	no changes
E32Q	no changes	L128I	no changes
E32H	no changes	L128V	no changes
E32N	no changes	E129Q	no changes
E33Q	no changes	E129H	no changes
E33H	no changes	E129N	no changes
E33N	no changes	D130N	no changes
Y35H	no changes	D130Q	no changes
Y35I	no changes	P133A	increase
P37A	no changes	P133S	no changes
P37S	no changes	R134H	increase
K38N	increase	R134Q	no changes
K38Q	no changes	F139I	no changes
E39Q	no changes	F139V	no changes
E39H	no changes	K140N	increase
E39N	no changes	K140Q	no changes
K41N	no changes	Y143H	no changes
K41Q	increase	Y143I	no changes
Y42H	increase	K145N	no changes
Y42I	increase	K145Q	no changes
F44I	no changes	F146I	no changes
F44V	no changes	F146V	no changes
L45I	no changes	D147N	increase
L45V	no changes	D147Q	increase
P48A	no changes	D153N	increase
P48S	no changes	D153Q	no changes
L52I	no changes	D154N	no changes
L52V	no changes	D154Q	no changes
F54I	no changes	L156I	increase
F54V	no changes	L156V	no changes
E56Q	increase	L157I	increase
E56H	no changes	L157V	no changes
E56N	increase	K158N	increase
P59A	no changes	K158Q	no changes
P59S	no changes	Y160H	no changes
P61A	no changes	Y160I	no changes
P61S	no changes	L162I	increase
R64H	no changes	L162V	no changes
R64Q	no changes	L163I	no changes
E65Q	increase	L163V	no changes
E65H	no changes	Y164H	no changes
E65N	no changes	Y164I	no changes
E66Q	increase	F166I	increase
E66H	no changes	F166V	no changes
E66N	no changes	R167H	increase
K70N	no changes	R167Q	increase

TABLE 2: Growth Hormone LEADS and SuperLEADs

Mutation	Resistance to proteolysis	Mutation	Resistance to proteolysis
K70Q	no changes	K168N	increase
L73I	no changes	K168Q	increase
L73V	increase	D169N	no changes
E74Q	no changes	D169Q	increase
E74H	no changes	M170I	no changes
E74N	increase	M170V	no changes
L75I	no changes	D171N	increase
L75V	no changes	D171Q	increase
L76I	no changes	K172N	no changes
L76V	no changes	K172Q	increase
R77H	no changes	E174Q	increase
R77Q	no changes	E174H	increase
L80I	no changes	E174N	increase
L80V	no changes	F176I	no changes
L81I	no changes	F176V	no changes
L81V	increase	L177I	increase
L82I	no changes	L177V	increase
L82V	no changes	R178H	no changes
W86H	no changes	R178Q	increase
W86S	no changes	R183H	no changes
L87I	no changes	R183Q	no changes
L87V	increase	E186Q	no changes
E88Q	no changes	E186H	no changes
E88H	no changes	E186N	no changes
E88N	no changes	F191I	increase
P89A	no changes	F191V	no changes
P89S	no changes	hGH WT	
F92I	no changes	hGH NIBSC	

3. Interferon gamma

Using the methods as claimed in the above-captioned application, 148 variants of interferon-gamma (IFN-gamma) were generated and tested based on the predetermined property of increased protease resistance. IFN-gamma candidate LEADs were tested *in vitro* for protease resistance by incubating 15 ng of native IFN-gamma or variants with a cocktail of proteases as described above, followed by assessment of residual anti-viral activity as described above. Table 3 shows the results of some of the tested polypeptides. The results show that many of the polypeptides tested exhibited an increased resistance to protease *in vitro* as compared to native IFN-gamma.

TABLE 3: Interferon-gamma LEAD Polypeptides

Mutation	Resistance to Proteolysis	Mutation	Resistance to Proteolysis	Mutation	Resistance to Proteolysis
D5N	no change	D44N	ND	D94N	ND
D5Q	no change	D44Q	ND	D94Q	ND
P6A	no change	R45H	no change	F95I	ND
P6S	no change	R45Q	no change	F95V	ND
Y7H	no change	F57I	no change	E96Q	Increase
Y7I	no change	F57V	increase	E96H	Increase
K9N	no change	K58N	no change	E96N	Increase
K9Q	no change	K58Q	no change	K97N	no change
E10Q	no change	L59I	no change	K97Q	no change
E10H	no change	L59V	no change	L98I	Increase
E10N	no change	F60I	no change	L98V	Increase
E12Q	no change	F60V	ND	Y101H	no change
E12H	no change	K61N	no change	Y101I	no change
E12N	no change	K61Q	no change	D105N	Increase
L14I	no change	F63I	increase	D105Q	Increase
L14V	no change	F63V	increase	L106I	Increase
K15N	no change	K64N	ND	L106V	Increase
K15Q	no change	K64Q	increase	E115Q	ND
K16N	no change	D65N	no change	E115H	ND
K16Q	no change	D65Q	no change	E115N	ND
Y17H	no change	D66N	increase	E122Q	ND
Y17I	no change	D66Q	increase	E122H	ND
F18I	no change	K71N	no change	E122N	ND
F18V	no change	K71Q	no change	L123I	ND
D24N	ND	E74Q	no change	L123V	ND
D24Q	ND	E74H	no change	P125A	Increase
D27N	ND	E74N	no change	P125S	Increase
D27Q	ND	K77N	no change	K128N	no change
L31I	ND	K77Q	no change	K128Q	no change
L31V	ND	E78Q	no change	K131N	ND
F32I	ND	E78H	no change	K131Q	ND
F32V	ND	E78N	no change	R132H	no change
L33I	increase	D79N	no change	R132Q	no change
L33V	increase	D79Q	ND	K133N	Increase
L36I	no change	K83N	no change	K133Q	Increase
L36V	no change	K83Q	no change	R134H	Increase
K37N	increase	F84I	ND	R134Q	Increase
K37Q	increase	F84V	ND	M137I	ND
W39H	ND	K89N	increase	M137V	Increase
W39S	ND	K89Q	increase	L138I	ND
K40N	no change	K90N	increase	L138V	Increase
K40Q	no change	K90Q	increase	F139I	Increase
E41Q	increase	K91N	ND	F139V	Increase
E41H	increase	K91Q	ND	R142H	Increase
E41N	increase	R92H	no change	R142Q	Increase
E42Q	no change	R92Q	no change	R143H	Increase
E42H	no change	D93N	ND	R143Q	Increase
E42N	no change	D93Q	ND		

ND: Not determined

4. Erythropoietin

Using the methods as claimed in the above-captioned application, 199 variants of erythropoietin (EPO) were generated and tested based on the predetermined property of increased protease resistance. EPO candidate LEADs were tested *in vitro* for protease resistance by incubating 557.2 ng of native EPO or variants with a cocktail of proteases containing a 1.5% protease mixture (wt/wt) as described above, followed by assessment of residual anti-proliferative activity on TF-1 cells as described above. Table 4 shows the results of some of the tested polypeptides. The results show that many of the polypeptides tested exhibited an increased resistance to protease *in vitro* as compared to native EPO.

In a second set of experiments, resistance to proteolysis was measured as described above, except that a higher concentration of proteases containing 3% protease mixture (wt/wt) containing each of the following proteases, α-chymotrypsin, Endoproteinase GluC and trypsin (Sigma), was used to assess resistance to proteolysis. The results are shown below for some of the variant polypeptides in Table 5. The data are expressed as relative resistance to proteases among the samples tested: (+), (++), or (+++), with (+++) indicating the highest resistance to proteases, and (-) indicating no change compared to native erythropoietin.

TABLE 4: Erythropoietin LEAD Polypeptides	
Mutation	**Resistance to proteases**
D43Q	ND
K45Q	no change
F48I	no change
F48V	increase
K52Q	increase
E55Q	no change
E55H	ND
E72Q	ND
E72H	ND
L75V	ND
L75I	increase
R76H	no change
R76Q	no change
P121S	ND
P121A	increase
P122S	increase
P122A	increase
D123Q	ND
P129S	increase
P129A	increase
L130V	ND
L130I	increase
R131H	increase
R131Q	increase

ND: Not determined

TABLE 5: Erythropoietin LEAD Polypeptides – Higher Protease Concentration	
Mutation	**Resistance to proteases**
D43Q	-
K45Q	-
F48I	++
F48V	+
K52Q	++
E55Q	-
E55H	-
E72Q	ND
E72H	ND
L75V	-
L75I	+
R76H	-
R76Q	-
P121S	-
P121A	+
P122S	+
P122A	+
D123Q	++
P129S	+
P129A	+
L130V	++
L130I	+++
R131H	+
R131Q	++

ND: Not determined

B. *In Vivo* Exposure

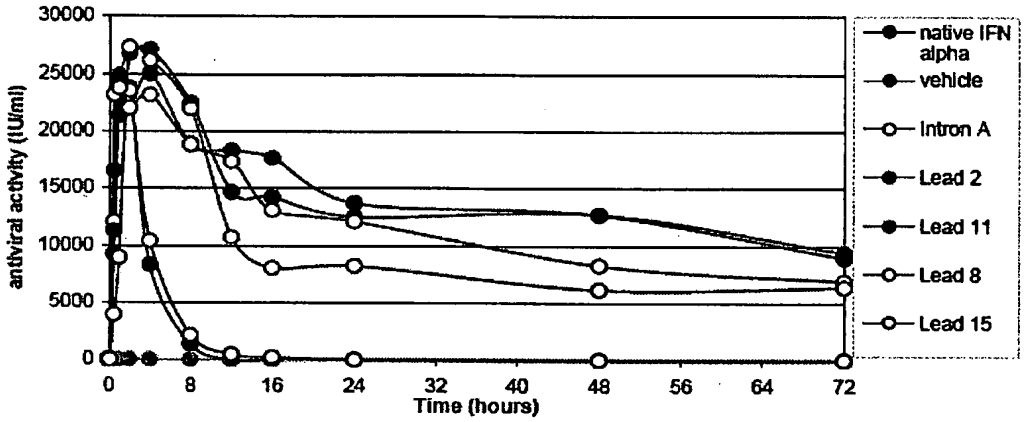
Selected IFN-α LEADs were assayed for their residual activity following exposure to proteases *in vivo* after subcutaneous or per-oral administration.

1. Subcutaneous

Four selected IFN-α2b LEADS identified by the method described in the above-captioned application, each containing a single amino acid change compared to native IFN-α2b, were tested for their residual activity in plasma following subcutaneous administration. The LEADs were assessed for their residual activity in serum following subcutaneous administration into Cynomolgus monkeys as compared to native IFN-α, including a native IFN-α sold commercially as Intron A®. After a single dose of 0.3 mg/kg of polypeptide, residual activity in

the plasma was determined at various time-points post-administration. The results in Figure 2 show that both native IFN-α and Intron A® retained anti-viral activity that was barely detectable 8 hours after administration, with no detectable activity observed at later time points. All of the IFN-α LEADs tested retained anti-viral activity up to the last time point tested at 72 hours post-injection, with little decrease in anti-viral activity observed up to 8 hours post-injection of the IFN-α LEAD molecules.

FIGURE 2

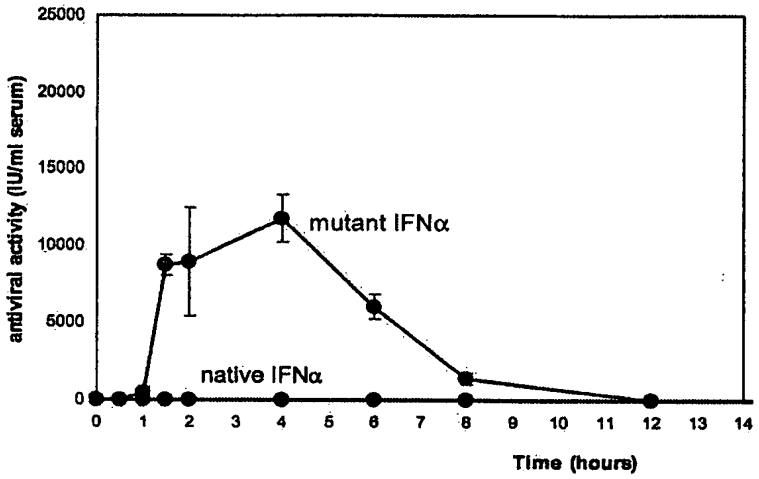


2. Oral Administration

A selected candidate mutant IFN-alpha (IFNα) LEAD containing a single amino acid change identified using the methods as claimed in the above-caption application was investigated for its residual activity after per-os (PO; by oral route) administration. After a single dose of 0.9 mg/kg of enteric-coated capsule formulation of mutant IFNα or native IFNα by PO administration in Cynomolgus monkeys, residual anti-viral activity was determined in the plasma to compare the pharmacokinetic (PK) and the systemic exposure profile of the two polypeptides after oral administration. The results are depicted in Figure 3 below. The PK profiles of mutant IFN-α and native IFN-α detected in the blood circulation by anti-viral activity assay are represented as the average of the male and female monkeys for each molecule. The data show that following oral administration of mutant IFN-α in enteric-coated capsules a significant anti-

viral activity was detected in monkey plasma, whereas no activity was detected in animals treated with native IFN-α at the same dose. The results evidence that a polypeptide, containing only a single mutation and identified using the methods as claimed in the above-captioned application, exhibits a greatly improved resistance to protease compared to the native polypeptide.

FIGURE 3



III. Conclusion

The results show that by using the method as claimed in the above-captioned application, candidate LEAD polypeptides are efficiently generated and tested for a predetermined property and activity, such as protease resistance. The results also show that the method reliably results in the identification of LEAD molecules, including those containing only a single mutation, exhibiting the predetermined property of protease resistance, thereby resulting in improved pharmacokinetic profiles following subcutaneous or per-oral administration. Hence, the results presented here exemplify the power and capability of the method described in the above-captioned application and its application to the discovery of unique therapeutic polypeptides.

* * *

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent resulting therefrom.

August 29 2007
Date

Manuel Vega